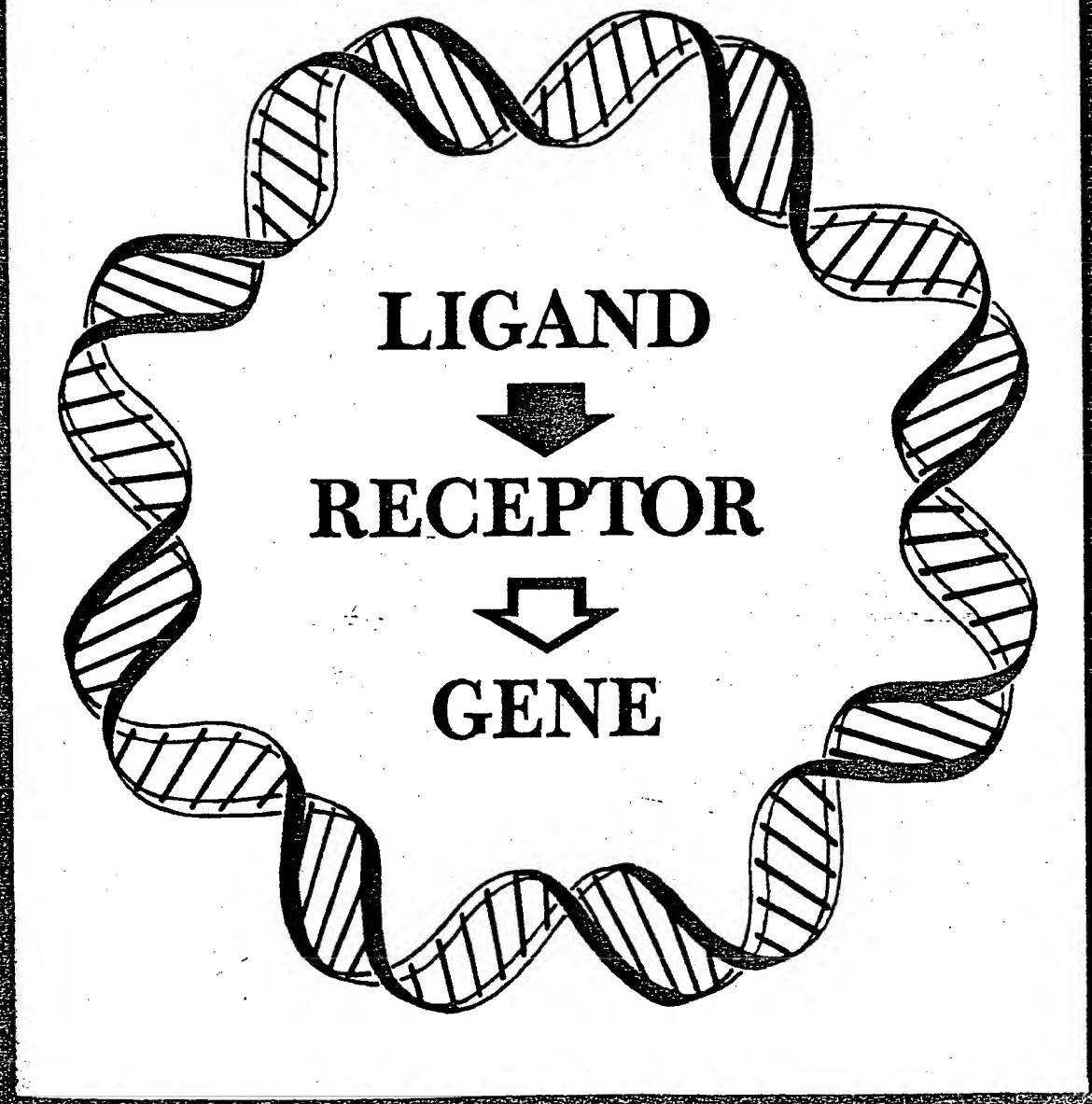
MOLECULAR ENDOCRINOLOGY

Volume 2, Number 12 December 1988



ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY

The Rat Androgen Receptor: Primary Structure, Autoregulation of its M sseng r Ribonucleic Acid, and Immunocytochemical Localization of the Receptor Protein

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A composite androgen receptor DNA sequence 4181 base pairs in length was determined from three cDNA clones isolated from a rat epididymal bacteriphage lambda gt11 library. An open reading frame of 902 amino acids encodes a protein of 98,227 mol wt. Structural domains characteristic of the steroid receptor family include an amino-terminal region with five repeated amino acid motifs, a central DNA-binding domain homologous with other steroid receptors, and a carboxyl-terminal steroid-binding region. A receptor cDNA probe used in Northern blot analysis hybridized with a predominant 10-kilobase androgen receptor mRNA in male reproductive tissues of the rat. Autoregulation of androgen receptor mRNA was indicated in rat ventral prostate by an increase in the level of 10-kilobase mRNA after castration and suppression of receptor mRNA upon androgen restimulation. A 15 amino acid peptide with sequence derived from the deduced androgen receptor sequence was synthesized and used as immunogen in raising receptor antibodies in rabbits. Antisera reacted with high titer against the synthetic peptide by enzyme-linked immunosorbent assay and against the native [^{3}H]dihydrotestosterone-labeled androgen receptor as evidenced by an increase in receptor sedimentation rate determined by sucrose gradient centrifugation. Immunocytochemical staining localized the androgen receptor t epithelial cell nuclei in rat ventral prostate. (Mol cular Endocrinology 12: 1276–1285, 1988).

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Molecular Endocrinology

INTRODUCTION

The recent cloning of steroid receptor cDNAs has revealed striking structural similarities among the receptor proteins suggesting that they are members of a closely related family. Amino acid sequence conservation particularly within the central DNA-binding domain places the receptor family within an even larger group of nuclear proteins which share a metal-binding motif believed to have a role in DNA binding (1). Consensus oligonucleotide probes with conserved features of steroid receptor DNA-binding domains facilitated the cloning of the last of the known steroid receptor genes, the human androgen receptor (hAR) (2, 3). The sequence of the AR DNA-binding domain confirmed that it is a member of the nuclear receptor family and that it shares greatest sequence homology with the progesterone, mineralocorticoid, and glucocorticoid receptors (2, 3).

Knowledge of AR DNA sequence has opened up important new methodologies for structure-function studies on the AR. From the deduced amino acid sequence, synthetic peptides can be used as immunogens to produce antibodies that recognize the native receptor protein. This approach has been used successfully to raise antibodies against the DNA-binding region for studies with progesterone (4), glucocorticoid (5), and estrogen receptors (6). An advantage of this approach is that antibodies are obtained against selected well defined regions of the protein.

To elucidate the structure of the rat AR, the entire coding region of the cDNA was cloned and sequenced. Hybridization analysis with AR cDNA probes demonstrated that androgen suppresses the steady state level of its own 10-kilobase (kb) receptor mRNA. From the deduced amino acid sequence, a peptide was synthe-

sized and used as immunogen to raise antibodies that recognize the native receptor protein.

RESULTS

Isolation and Sequencing of Rat AR cDNA Clones

A 1.5-kilobase pairs (kbp) *Eco*RI fragment of hAR cDNA, ARHEL1 (2) was used to screen 200,000 recombinant plaques of a lambda gt11 rat epididymal cDNA library. One positive clone (rARep1) was identified and plaque purified. Electrophoretic analysis after digestion with *Eco*RI revealed two fragments of approximately 1 and 2 kbp, indicating a total insert length of approximately 3 kbp and the presence of an internal *Eco*RI site. From the position of the conserved DNA-binding domain (see below), it was determined that clone rARep1 contains primarily sequence 5′ to the DNA-binding domain (Fig. 1A). Clone rARep1 and a 3′ hAR DNA probe (436 base pairs (bp) *Eco*RI-*Pst*I fragment of ARHFL1) (2) were used together to screen an additional 800,000 recombinants of the same amplified rat epididymal cDNA library. Five additional positive clones were identified of which one was fully characterized. This second clone, rARep2, contained a 1.7-kbp insert and included the DNA-binding domain and 3′-coding sequence (Fig. 1A).

The cDNA clones rARep1 and rARep2 were sequenced from the cloned restriction fragments diagrammed in Fig. 1B. Their composite sequence (Fig. 2) indicates a predicted methionine initiation codon at nucleotide 994 which begins an open reading frame of 902 amino acids, ending at nucleotide 3700 with the stop codon TGA. The next upstream methionine (ATG) occurs at nucleotide residue 578 and is followed immediately by an in-frame stop codon at residue 602 (shown underlined in Fig. 2). An additional stop codon (TAA) occurs at residue 988 in-frame with the coding sequence directly before the predicted initiation methionine (shown underlined in Fig. 2). The mol wt of the protein derived from the nucleotide sequence 98,227, is somewhat lower than the mol wt of the native AR as determined from hydrodynamic measurements (117,000) (7). The DNA-binding domain contains nine cysteine residues (each marked with an asterisk in Fig. 2), is centrally located in the molecule, and shows a high degree of homology with other members of the steroid receptor family (2, 3). Several stretches of repeated amino acids occur dispersed in the 5′-portion of the sequence, including five arginines, 22 glutamines, seven closely positioned prolines, six alanines, and six glycines (shown underlined in Fig. 2). The significance of these regions is not known but similar stretches of repeated amino acids have been observed in the hAR (8) and in other steroid receptors (9–11).

The 3′-sequence of an additional clone (rARep3) had 45(A) residues preceding the *Eco*RI linker sequence and thus extended the 18(A) residues present at the 3′-end of rARep2 (Fig. 2). The significance of this poly(A) region is not known, but undoubtedly it served as template for oligo d(T)-primed first-strand synthesis during preparation of the cDNA. It is unlikely that the stretch of (A) residues represents the poly(A)-addition sequence of a truncated androgen receptor mRNA; however, this possibility has not been ruled out. The poly(A) addition-like signal sequence that occurs at nucleotide 4129 (AAGAAA) does not conform to the consensus signal sequence (AATAAA), but has been shown to function as a poly(A)-addition signal with low efficiency (12, 13).

AR mRNA

The ^{32}P-labeled 1-kb *Eco*RI fragment of rARep1 was used as a hybridization probe to examine the tissue distribution of AR mRNA. The 10-kb mRNA shown

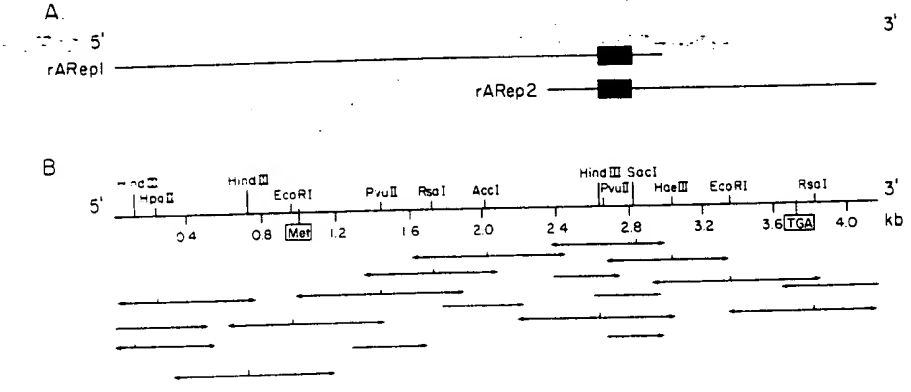

Fig. 1. Sequencing Strategy for rat AR cDNA

A, Schematic diagram of two cDNA clones, rARep1, 3.0 kbp and rARep2, 1.7 kbp, isolated from a rat epididymal lambda gt11 cDNA library. The DNA-binding region is indicated by ■. B, Restriction fragments used in sequencing. *Numbers below the line* refer to kilobase pairs from the 5′-end of the composite cDNA. *Arrows* indicate the direction and extent of sequencing 5′ to 3′ on the upper and lower strands. Both strands of the two cDNA clones were completely sequenced. The starting methionine and stop codon (TGA) are enclosed in *boxes*.

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ATCCCTAGGAGCCAGCCTGCTGGGAGAACCAGAGGGTCCGGAGCAAACCTGGAGGCTGAGAGGGCATCAGAGGGGAAAAGACTGAGTTAGCCACTCCAGTGCCATACAGAAGCTTAAGGG  120
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GTAGTCGAAAGCAAAACAAAAGCAAAAAGAAAACAAAAAGAAAATAGCCCAGTTCTTATTTGCACCTGCTTCAGTGGACATTGACTTTGGAAGGCAGAGAATTTTCCTTCCCCCCAGTCA  720
AGCTTTGAGCATCTTTTAATCTGTTCTTCAAGTATTTAGGGACAAACTGTGAAACTAGCAGGGCAGATCCTGTCTAGCGCGTGCCTTCCTTTACAGGAGACTTTGAGGCTATCTGGGCGC  840
TCCCCCCCCTCCCTGCAAGTTTTCTTCCCTGGAGCTTCCCGCAGGTGGGCAGCTAGCTGCAGATACTACATCATCAGTCAGTAGAACTCTTCAGAGCAAGAGACGAGGAGGCAGGATAAG  960

                                                                                                                    10                  20
GGAATTCGGTGGAAGCTAGAGACAAGCTAAAGGATGGAGGTGCAGTTAGGGCTGGGAAGGGTCTACCCACGGCCCCCGTCCAAGACCTATCGAGGAGCGTTCCAGAATCTGTTCCAGAGC 1080
                                  MetGluValGlnLeuGlyLeuGlyArgValTyrProArgProProSerLysThrTyrArgGlyAlaPheGlnAsnLeuPheGlnSer
                                                                                                                    60
 30                  40                  50
GTGCGCGAAGCGATCCAGAACCCGGGCCCCAGGCACCCTGAGGCCGCTAGCATAGCACCTCCCGGTGCCTGTTTACAGCAGCGGCAGGAGACTAGCCCCCGGCGGCGGCGGCGGCAGCAG 1200
ValArgGluAlaIleGlnAsnProGlyProArgHisProGluAlaAlaSerIleAlaProProGlyAlaCysLeuGlnGlnArgGlnGluThrSerProArgArgArgArgArgGlnGln
 70                  80                  90                  100
CACCCTGAGGATGGCTCTCCTCAAGCCCACATCAGAGGCACCACAGGCTACCTGGCCCTGGAGGAGGAACAGCAGCCTTCACAGCAGCAGTCAGCCTCCGAGGGCCACCCTGAGAGCGGC 1320
HisProGluAspGlySerProGlnAlaHisIleArgGlyThrThrGlyTyrLeuAlaLeuGluGluGluGlnGlnProSerGlnGlnGlnSerAlaSerGluGlyHisProGluSerGly
110                 120                 130                 140
TGCCTCCCGGAGCCTGGAGCTGCCACGGCTCCTGGCAAGGGGCTGCCGCAGCAGCCACCAGCTCCTCCAGATCAGGATGACTCAGCTGCCCCATCCACGTTGTCCCTACTGGGCCCCACT 1440
CysLeuProGluProGlyAlaAlaThrAlaProGlyLysGlyLeuProGlnGlnProProAlaProProAspGlnAspAspSerAlaAlaProSerThrLeuSerLeuLeuGlyProThr
150                 160                 170                 180
TTCCCAGGCTTAAGCAGCTGCTCCGCAGACATTAAAGACATCCTGAGCGAGGCCGGCACCATGCAACTTCTTCAGCAGCAGCAGCAACAGCAACAGCAGCAGCAGCAGCAGCAGCAG 1560
PheProGlyLeuSerSerCysSerAlaAspIleLysAspIleLeuSerGluAlaGlyThrMetGlnLeuLeuGlnGlnGlnGlnGlnGlnGlnGlnGlnGlnGlnGlnGlnGlnGln
190                 200                 210                 220
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230                 240                 250                 260
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AspSerAlaLysGluLeuCysLysAlaValSerValSerMetGlyLeuGlyValGluAlaLeuGluHisLeuSerProGlyGluGlnLeuArgGlyAspCysMetTyrAlaSerLeuLeu
270                 280                 290                 300
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GlyGlyProProAlaValArgProThrProCysAlaProLeuAlaGluCysLysGlyLeuSerLeuAspGluGlyProGlyLysGlyThrGluGluThrAlaGluTyrSerSerPheLys
310                 320                 330                 340
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350                 360                 370                 380
GACGAGGCAGCAGCATACCAGAATCGCGACTACTACAACTTTCCGCTCGCTCTGTCCGGGCCGCCGCACCCCCCGCCCCCTACCCATCCACACGCCCGCATCAAGCTGGAGAACCCGTCG 2160
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390                 400                 410                 420
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AspTyrGlySerAlaTrpAlaAlaAlaAlaAlaAlaGlnCysArgTyrGlyAspLeuAlaSerLeuHisGlyGlySerValAlaGlyProSerThrGlySerProProAlaThrAlaSerSer
430                 440                 450                 460
TCCTGGCATACTCTCTTCACAGCTGAAGAAGGCCAATTATATGGGCCAGGAGGCGGGGGCGGCAGCAGTAGCCCAAGCGATGCTGGGCCTGTAGCCCCCTATGGCTACACTCGGCCCCCT 2400
SerTrpHisThrLeuPheThrAlaGluGluGlyGlnLeuTyrGlyProGlyGlyGlyGlyGlySerSerSerProSerAspAlaGlyProValAlaProTyrGlyTyrThrArgProPro
470                 480                 490                 500
CAGGGGCTGGCAAGCCAGGAGGGTGACTTCTCTGCCTCTGAAGTGTGGTATCCTGGTGGAGTTGTGAACAGAGTCCCCTATCCCAGTCCCAGTTGTGTTAAAAGTGAAATGGGACCTTGG 2520
GlnGlyLeuAlaSerGlnGluGlyAspPheSerAlaSerGluValTrpTyrProGlyGlyValValAsnArgValProTyrProSerProSerCysValLysSerGluMetGlyProTrp
510                 520                 530                 540
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550                 560                 570                 580
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590                 600                 610                 620
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630                 640                 650                 660
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SerAlaGlySerProThrGluAspProSerGlnLysMetThrValSerHisIleGluGlyTyrGluCysGlnProIlePheLeuAsnValLeuGluAlaIleGluProGlyValValCys
670                 680                 690                 700
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AlaGlyHisAspAsnAsnGlnProAspSerPheAlaAlaLeuLeuSerSerLeuAsnGluLeuGlyGluArgGlnLeuValHisValValLysTrpAlaLysAlaLeuProGlyPheArg
710                 720                 730                 740
AACTTGCATGTGGATGACCAGATGGCAGTCATTCAGTATTCCTGGATGGGACTGATGGTATTTGCCATGGGTTGGCGGTCCTTCACTAATGTCAACTCTAGGATGCTCTACTTTGCACCT 3240
AsnLeuHisValAspAspGlnMetAlaValIleGlnTyrSerTrpMetGlyLeuMetValPheAlaMetGlyTrpArgSerPheThrAsnValAsnSerArgMetLeuTyrPheAlaPro
750                 760                 770                 780
GACCTGGTTTTCAATGAGTATCGCATGCACAAGTCTCGAATGTACAGCCAGTGCGTGAGGATGAGGCACCTTTCTCAAGAGTTTGGATGGCTCCAGATAACCCCCCAGGAATTCCTGTGC 3360
AspLeuValPheAsnGluTyrArgMetHisLysSerArgMetTyrSerGlnCysValArgMetArgHisLeuSerGlnGluPheGlyTrpLeuGlnIleThrProGlnGluPheLeuCys
790                 800                 810                 820
ATGAAAGCACTGCTACTCTTCAGCATTATTCCAGTGGATGGGCTGAAAAATCAAAAATTCTTTGATGAACTTCGAATGAACTACATCAAGGAACTTGATCGCATCATTGCATGCAAAAGA 3480
MetLysAlaLeuLeuLeuPheSerIleIleProValAspGlyLeuLysAsnGlnLysPhePheAspGluLeuArgMetAsnTyrIleLysGluLeuAspArgIleIleAlaCysLysArg
830                 840                 850                 860
AAAAATCCCACATCCTGCTCAAGGCGCTTCTACCAGCTCACCAAGCTCCTGGATTCTGTGCAGCCTATTGCAAGAGAGCTGCATCAATTCACTTTTGACCTGCTAATCAAGTCCCATATG 3600
LysAsnProThrSerCysSerArgArgPheTyrGlnLeuThrLysLeuLeuAspSerValGlnProIleAlaArgGluLeuHisGlnPheThrPheAspLeuLeuIleLysSerHisMet
870                 880                 890                 900
GTGAGCGTGGACTTTCCTGAAATGATGGCAGAGATCATCTCTGTGCAAGTGCCCAAGATCCTTTCTGGGAAAGTCAAGCCCATCTATTTCCACACACAGTGAAGATTTGGAAACCCTAAT 3720
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ACCCAAACCCACCTTGTTCCCTTTTCAGATGTCTTCTGCCTGTTATATAACTCTGCACTACTTCTCTGCATTGCCTTGGGGGAAATTCCTCTACTGATGTACAGTCTGTCATGAACATGT 3840
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GTTTAGAGAGCTATAAGTATCTTGGGAAGAAACAAACAGAGAGAGTAAAAAAACC(A)45
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Fig. 2. Nucleotide and Predicted Amino Acid Sequence of rat AR cDNA

The sequence shown begins 120 bp downstream from the 5′-end of cDNA clone rARep1 (see Fig. 1). The open reading frame begins at nucleotide 994 and ends with a stop codon TGA at nucleotide 3700. Underlined is a methionine codon (ATG, nucleotide residue 578) that immediately precedes a stop codon (TAG, nucleotide residue 602), and both of these precede the predicted methionine initiation codon (ATG, nucleotide residue 994). Also underlined is a stop codon (TAA) in reading frame at residue 988, 5′ to the predicted initiation methionine at residue 994. Several amino acid repeat sequences are underlined as is a sequence resembling a poly(A) addition signal at nucleotide 4129 (AAGAAA). On the 3′-end of the cDNA insert rARep3 are 45 adenylic acid residues which begin 23 nucleotides after the poly(A) addition-like signal. An *asterisk* highlights each of the nine conserved cysteines of the DNA-binding domain. The sequence of amino acids enclosed in a *box* was used in peptide synthesis for polyclonal antibody production. The nucleotide sequence of the rat AR is available from GenBank under accession number M20133.

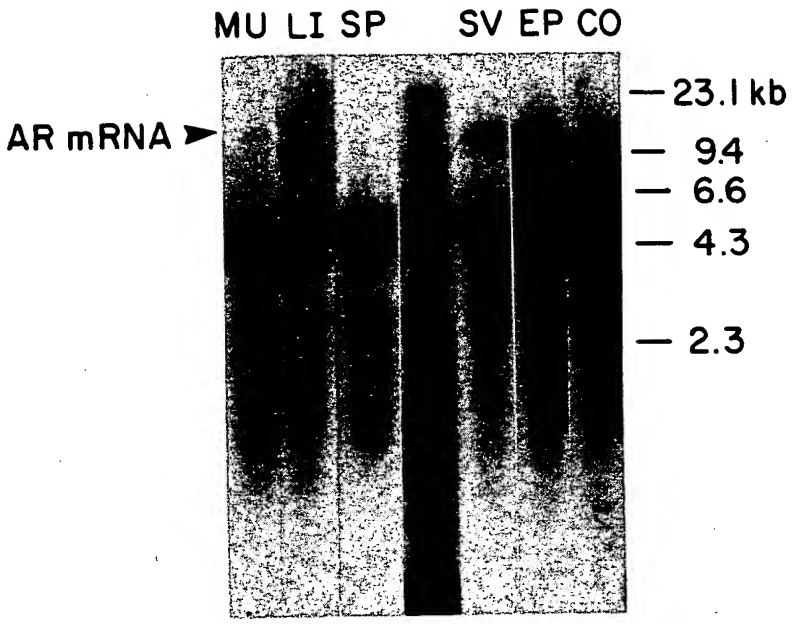

Fig. 3. Northern Blot Analysis of AR mRNA in Rat Tissues

Aliquots of total RNA (20 μg) isolated from tissues of intact adult rats were analyzed by blot hybridization using as probe a ^{32}P-labeled rat AR 1-kbp 5′-fragment of rARep1. RNA samples are shown from skeletal muscle (MU), liver (LI), spleen (SP), seminal vesicle (SV), epididymis (EP), and coagulating gland (CO). Molecular weight markers in the middle lane were ^{32}P-labeled, *Hin*dIII-digested lambda DNA and *Hae*III-digested phi X 174 DNA.

previously to encode the AR (2) was found as a predominant hybridizing band in total RNA isolated from rat liver, seminal vesicle, epididymis and coagulating gland (anterior prostate) (Fig. 3). A low level of AR mRNA was detected in extracts of muscle. Receptor mRNA was undetectable in spleen, a tissue shown previously to lack AR steroid-binding activity (14). The radioactive bands 5 kb and smaller likely resulted from nonspecific cross-hybridization since they coincide with ribosomal RNA and are of lower intensity when other AR hybridization probes are used. These bands are present also in spleen, a tissue presumed to lack AR mRNA, and the predominant 5-kb band is observed when rat ribosomal RNA is analyzed on a similar Northern blot. Of the tissues examined, highest AR mRNA levels were in epididymis (Fig. 3) and ventral prostate (see below).

Androgen dependence of receptor mRNA was investigated by comparing steady state levels of AR mRNA in rat ventral prostate after androgen withdrawal, with and without androgen replacement. Total RNA was isolated 24 and 48 h after androgen withdrawal and analyzed by Northern blot hybridization. Equivalent loading of total RNA (20 μg) on each lane of the gel was verified by subsequent hybridization of the blot with a ^{32}P-labeled 0.8-kbp chicken B-actin cDNA probe (Oncor, Gaithersburg, MD). AR mRNA levels increased 2.5-fold within 24 h after castration and remained elevated for at least 48 h (Fig. 4, lanes 2–4). Androgen administration at 24 h after castration reduced receptor mRNA 24 h later to a level below that of the intact control animal (64% of control) (Fig. 4, lane 5). Thus androgen acts to repress the steady state level of its receptor mRNA. Preliminary studies using cycloheximide treatment to evaluate mRNA turnover suggest that the apparent androgen-induced decrease in receptor mRNA levels does not result from a significant change in the mRNA half-life.

An AR Antibody

A peptide with sequence derived from a 15 amino acid region common to the rat AR and hAR was synthesized and used to raise antibodies that would have the potential of reacting with native AR. The sequence shown enclosed in a box in Fig. 2 is immediately 5′ to the DNA-binding domain and is unique to the AR based on available steroid receptor sequence information. An additional criterion for peptide selection was its hydrophilicity as determined from a hydropathic plot of the AR sequence (8). It was predicted that the region selected for peptide synthesis would extend to the hydrophilic exterior and that contiguous proline residues might contribute secondary structure and thus increase peptide antigenicity.

The 15 amino acid AR sequence and three linker amino acids, Gly-Gly-Cys, were synthesized (peptide 875) and covalently coupled to Keyhole limpet hemocyanin for use in antibody production. The antisera demonstrated high titer reactivity toward peptide 875 in enzyme-linked immunosorbent assay (ELISA) (Fig. 5). The titer increased with each booster injection,

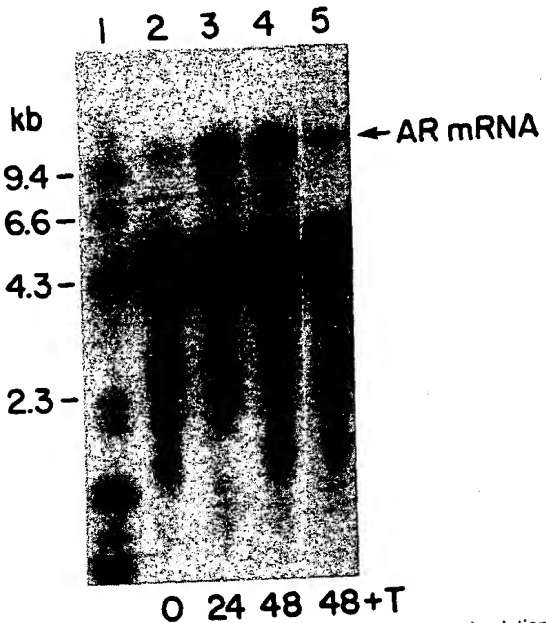

Fig. 4. Influence of Androgen Withdrawal and Restimulation on Steady State Levels of AR mRNA in Rat Ventral Prostate

RNA was isolated from ventral prostates of adult Sprague Dawley rats killed in groups of four to five each either after no treatment (intact controls, lane 2), 24 h (lane 3) or 48 h (lane 4) after castration. An additional group of animals was administered one injection sc of 2 mg testosterone propionate (T) in sesame oil 24 h after castration and 24 h before killing (lane 5). Aliquots of total RNA (20 μg) were analyzed on Northern blots using a ^{32}P-labeled rat AR 1-kbp 5′ *Eco*RI fragment of rARep1 as probe. Molecular weight markers in lane 1 were ^{32}P-labeled, *Hin*dIII-digested lambda DNA and *Hae*III-digested phi X 174 DNA. The autoradiogram was scanned with a densitometer to determine absolute changes in mRNA levels.

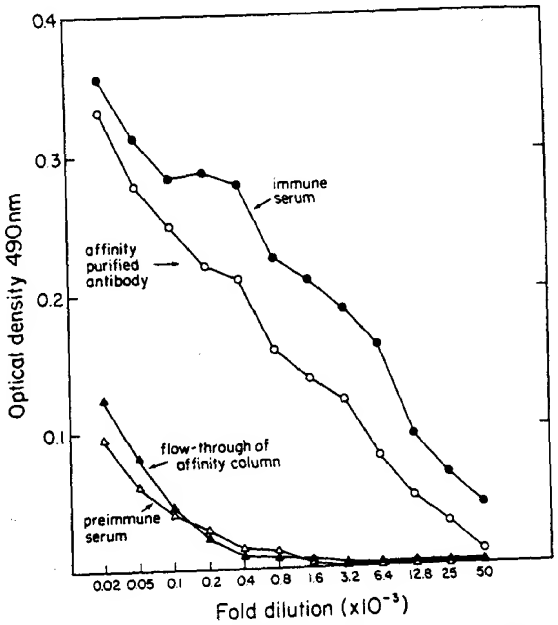

Fig. 5. ELISA Assay of Preimmune and Antiserum Preparations

Serial dilutions of antibody preparations were tested by ELISA including immune serum from the first bleeding of rabbit AR1-52 (●), antibody (1.5 mg/ml protein) obtained from affinity chromatography on a peptide 875-Sepharose column (○), flow-through fraction of the 875 peptide-Sepharose column (▲), and preimmune serum collected from rabbit AR1-52 before immunization (△). Uncoupled peptide 875 (5 pmol) was fixed to the bottom of 96-well microtiter plates with methanol, blocked for 1 h with 3% BSA in PBS, and incubated for 2 h with primary antibody, followed by a 2-h incubation with goat anti-rabbit horse radish peroxidase (Calbiochem, San Diego, CA). After washing, color development was with a *O*-phenylenediamine solution. Optical density was determined on a microtiter plate reader at 490 nm.

showing reactivity against peptide 875 at dilutions greater than 25,000. Antigen-affinity chromatography resulted in a 10-fold increase in activity per mg protein. The flow-through fraction of the peptide-affinity column, as well as preimmune serum, showed little reactivity by ELISA (Fig. 5).

Antiserum reactivity with native AR was investigated by sucrose gradient centrifugation. Antibodies obtained by antigen-affinity chromatography caused the 4.5S [^{3}H]dihydrotestosterone-labeled AR to sediment at 10S (Fig. 6) No peak of radioactivity was observed when cytosol was incubated with [^{3}H]dihydrotestosterone and a 100-fold excess unlabeled dihydrotestosterone. With further antibody dilution, the receptor sedimented at 7S (data not shown), suggesting that at high antibody concentrations, more than one antibody molecule may take part in receptor-antibody complex formation. Identical results were observed using the IgG fraction obtained from protein A-agarose chromatography of rabbit antiserum. Preincubation of the antiserum preparations with peptide 875 prior to combining with [^{3}H]dihydrotestosterone-labeled receptor eliminated the reactive antibodies as measured by ELISA and abolished the increased sedimentation of the 4.5S receptor. Neither preimmune serum nor the immunoglobulin G (IgG) fraction of preimmune serum had any effect on receptor sedimentation (data not shown). The titer of crude antiserum obtained from the first immune bleeding was such that a 1:10 dilution of antiserum increased the sedimentation of the 4.5S [^{3}H]methyltrienolone-labeled receptor to 10S and a 1:500 dilution to 7S. Dilution of antiserum to 1:1000 resulted in loss of any significant change in receptor sedimentation. Studies designed to establish the specificity of the antibody indicated a lack of cross-reaction with other steroid receptors. No cross-reactivity was observed with the chick progesterone receptor by western blot analysis (Smith, David, and David Toft, personal communication) or with the rat uterine estrogen receptor by western blot or sucrose gradient analysis (Korach, Kenneth, personal commu-

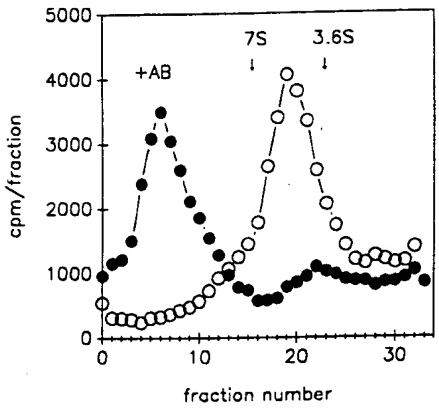

Fig. 6. Sucrose Gradient Analysis of the AR after Incubation with 875 Polyclonal Antibody

A, Dunning tumor AR fraction prepared as described in *Materials and Methods* was incubated for 3 h at 0 C with (●) or without (○) an immunoglobulin fraction of immune serum purified on a peptide-affinity column (150 μg protein in 0.1 ml). Sucrose gradient centrifugation was performed as described in *Materials and Methods*. The migration positions of the molecular weight markers ovalbumin (3.6S) and γ-globulin (7S) were determined as previously described (52).

nication). Since none of the other steroid receptors contained sequence similar to that of peptide 875, lack of cross-reaction was not unexpected.

Rabbit antiserum obtained with peptide 875 immunogen was used in immunocytochemical localization of the androgen receptor in rat ventral prostate. Immunostaining was confined largely to nuclei of epithelial cells, with little or no apparent staining in nuclei of stromal cells (Fig. 7B). This predominant localization to epithelial cells may be more characteristic of sexually mature rat prostate than of developing fetal prostate. Antibody titers increased with subsequent boostings of the rabbit when tested immunocytochemically and were effective at high dilution only after the third and fourth bleedings. Immunostaining of prostate epithelial cell nuclei was seen using third and fourth bleeding antisera at a dilution of 5,000 to 10,000 or with 1 to 10 μg/ml of the IgG fraction obtained by chromatography of immune serum on protein A-agarose. Control prostate sections incubated with the IgG fractions of preimmune serum (Fig. 7A) or immune serum absorbed with peptide 875 (1 μg peptide/ml containing 10 μg IgG) (Fig. 7C) were devoid of nuclear immunostaining. The absence of staining in controls indicates that the antibody interaction is specific. Similar treatment of frozen sections of spleen, a tissue that does not express androgen receptor as determined by measurements of [^{3}H]dihydrotestosterone binding or androgen receptor cDNA hybridization to a 10-kb mRNA species, showed no apparent staining with the antiserum (data not shown). Thus, rabbit antibodies raised to a synthetic peptide derived from the coding sequence of androgen receptor DNA react with rat AR both in a cell-free system and in fixed tissue sections.

DISCUSSION

AR cDNA is the last of the known mammalian steroid hormone receptors to be cloned. DNA sequence analysis indicates it has the same functional domain structure as other steroid hormone receptors. The NH_2-terminal region, characteristically variable among steroid receptors, shares little identity with other receptor sequences (8). The function of this N-terminal region is not fully understood though it is thought to have a role in transcriptional regulation or in targeting specific gene activation (15). A highly conserved DNA-binding domain, which is a hallmark of the superfamily of nuclear receptors, shares greatest homology with the progesterone, mineralocorticoid, and glucocorticoid receptors (2). Finally, the steroid binding domain is located in the carboxyl-terminal region as recently demonstrated (2). Rat AR and hAR share complete sequence identity in their DNA binding and steroid-binding domains. Detailed structural comparisons of the receptors in these species appear in a companion paper (8).

The apparent down-regulation of AR mRNA by its own ligand is a response previously observed in other steroid receptor systems. Dexamethasone has been shown to reduce the steady state level of its receptor mRNA by about 50% in human IM-9 lymphocytes and in rat pancreatic acinar AR42J cells. The mechanism of this suppression involved a decrease in the rate of gene transcription rather than a decrease in the half-life of the mRNA (16). In another study dexamethasone treatment of human HT1080 fibroblasts caused a 2- to 3-fold reduction in glucocorticoid receptor mRNA (17). Similarly diethylstilbestrol treatment of hormone withdrawn chicks caused a rapid decline in estrogen receptor mRNA, while progesterone increased the level of estrogen receptor mRNA (18). A rapid reduction of progesterone receptor mRNA levels by progesterone treatment has been observed in human breast cancer T47D cells (19). Thus autologous regulation of steroid receptor mRNAs characterized by steroid hormone-induced reduction of steady state mRNA levels appears to be a phenomenon common to members of this gene family.

While most hormone-regulated mRNAs in rat ventral prostate are stimulated by androgen, castration-stimulated and androgen-suppressed mRNAs have been identified, including the protooncogene c-*myc* (20) and proteins thought to be involved in prostate regression (21–23). The mechanism of androgen-induced suppression is not known, but may involve interference with transcription factors by receptor-protein and/or receptor-DNA interactions at a negative regulatory region as recently suggested by studies with other steroid receptors (24, 25). It is noteworthy with regard to localization of the AR in rat ventral prostate epithelial cells that these cells undergo more rapid and pronounced involutional changes after castration than do stromal cells (26–28).

Despite efforts in numerous laboratories to purify the AR, sufficient amounts of protein for antibody produc-

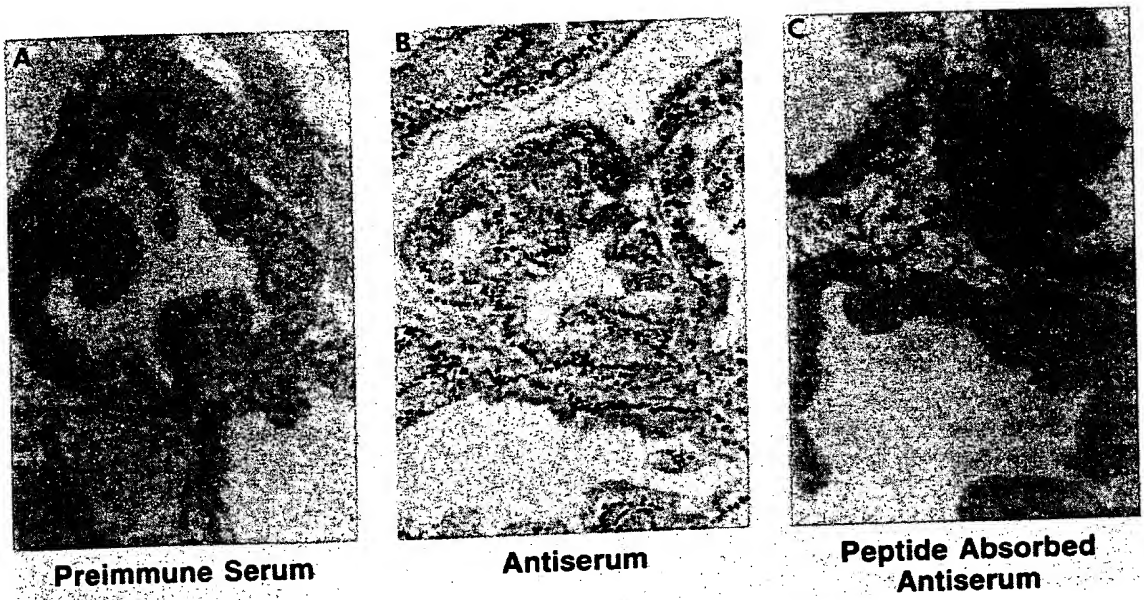

Fig. 7. Immunocytochemical Localization of the AR in Rat Ventral Prostate

Frozen sections of ventral prostate from intact adult rats were fixed in 4% paraformaldehyde, incubated with the IgG fraction of preimmune serum, 10 μg/ml (A), the IgG fraction of immune serum, 10 μg/ml (B), and the IgG fraction of immune serum 10 μg/ml, preabsorbed with 1 μg immunogen, peptide 875 (C), and stained by the avidin-biotin peroxidase method as described (51). The IgG fraction was purified by protein A-agarose chromatography. Immunostained sections were counterstained with 0.1% aniline blue. Thickness: 6 μm. Magnification, ×130.

tion have not been available. One approach attempting to circumvent this problem was to obtain from patients with prostatic disease autoimmune antibodies that react with the AR (29, 30). Derivation of receptor amino acid sequence from its cDNA has made it possible to identify, for purposes of peptide synthesis, potentially antigenic regions unique to the AR. Using this approach we have raised DNA-binding domain antibodies that cross-react with other native steroid receptors (4–6) as well as antibodies selective for the AR. To obtain specific AR antibodies, a peptide sequence outside the DNA-binding domain was chosen as antigen. The success of this approach was judged by antisera reactivity with the native AR. This approach to antibody production should be effective in further probing structure-function relationships of specific domains of steroid receptors in general.

Immunocytochemical studies using peptide-induced antibody demonstrate striking localization of AR to rat ventral prostate epithelial cell nuclei. Specificity of the antibody interaction is supported by lack of staining with preimmune antiserum and by the ability of peptide antigen to block the antibody-receptor interaction. The low amount of immunostaining in stromal cells of rat ventral prostate is somewhat surprising; however, biochemical evidence indicates that the epithelial cell content of AR is considerably greater than that of stroma in ventral prostate of sexually mature rats (31). Autoradiographic studies using radiolabeled androgen have similarly localized androgen receptor to nuclei (32) of prostate epithelial cells with little detectable receptor in stromal cells (33). The requirement of stromal mesenchyme for androgen-induced development of prostate epithelium from urogenital sinus (34, 35) argues for an action of androgen on stroma and thus for the presence of its receptor in stromal cells during early fetal development. Further studies using immunocytochemical detection of the AR at different times during development and after various hormone manipulations will be of great interest.

MATERIALS AND METHODS

Complementary DNA Cloning and Sequencing

A rat epididymal lambda gt11 expression library was prepared as previously described (36). RNA was isolated from epididymides of mature Sprague Dawley rats using guanidine thiocyanate extraction (37) and enriched in poly(A) RNA by chromatography on oligo-dT cellulose (38). Double stranded cDNA was prepared (39) and methylated with *Eco*RI methylase (40). Recombinants (200,000) of the amplified rat epididymal library (1.8×10^9 pfu/ml) were screened according to standard procedures (41) using a nick-translated 1.5-kbp human epididymal AR cDNA probe (ARHEL1) that contained the conserved DNA-binding domain (2). An additional 800,000 recombinants were rescreened from the same library using a 3′ hAR cDNA fragment (436-bp *Eco*RI-*Pst*I of ARHFL1) and a rat cDNA isolate (rARep1). Recombinant phage DNAs obtained after plaque purification were isolated from liquid lysates (42), digested with *Eco*RI, and the inserts isolated by agarose gel electrophoresis. Restriction fragments subcloned into bacteri-

ophage M13mp18 or M13mp19 were sequenced by the dideoxy chain termination method (43).

Analysis of RNA

In studies on androgen dependence of receptor mRNA, adult male Sprague Dawley rats (Zivic Miller, Zelienople, PA) were castrated through an abdominal incision under anesthesia (acepromazine-ketamine, 1:1 mixture, 0.1 ml/100 g BW). Ventral prostate and other tissues were rapidly removed from intact or castrated animals and immediately frozen in liquid nitrogen. Total RNA was isolated from tissues by centrifugation of a guanidine thiocyanate extract through 5.7 M cesium chloride as previously described (37). RNA extracts were denatured with glyoxal and dimethyl sulfoxide for 1 h at 50 C (44) and size fractionated on 1% agarose gels containing 10 mM sodium phosphate, pH 6.8. RNA was transferred to 0.2-μm nylon membranes (Biotrans, ICN, Irvine CA) (45) and cross-linked by brief exposure to UV light (46). DNA fragments used for hybridization were prepared, labeled to high specific activity with ^{32}P by nick translation, and used in hybridization reactions as previously described (20).

Antiserum Preparation

Peptide 875 used for immunization has the sequence Asp-His-Val-Leu-Pro-Ile-Asp-Tyr-Tyr-Phe -Pro-Pro-Gln-Lys-Thr-Gly-Gly-Cys. It was synthesized by Dr. David Klapper, Department of Microbiology, University of North Carolina at Chapel Hill (Chapel Hill, NC) on a Biosearch Model 9500 peptide synthesizer using tBOC chemistry (47). The last three carboxyl-terminal amino acids were included as a spacer. The peptide was coupled to Keyhole limpet hemocyanin through the cysteine residue using m-maleimido-benzoyl-*N*-hydroxysuccinimide ester (48) and through the lysine residue using glutaraldehyde (49). Two New Zealand White adult female rabbits were immunized (50) and sera titered against the free peptide by ELISA as previously described (Ref. 4, method 2). The ELISA titer increased with each bleeding from both rabbits, showing reactivity against the immunizing peptide at dilutions greater than 25,000. The rabbit serum showing the highest titer, 875-52, was used in these studies.

Antiserum was fractionated before reaction with the AR. In immunocytochemical studies, the immunoglobulin fraction was enriched in IgG by chromatography on protein A-agarose as previously described (51). For sucrose gradient analysis, the antiserum was either fractionated on protein A-agarose or on a peptide affinity column. Peptide 875 was covalently coupled to CNBr-activated Sepharose according to manufacturer's recommendations (Pharmacia, Piscataway, NJ). Antiserum (6 ml) from the first immune bleeding was chromatographed and peptide-specific antibodies eluted in 0.2 M glycine, 0.5 M KCl, pH 2.5. The optical density peak was pooled and immediately neutralized to pH 7.4 with 1 M Trizma Base and concentrated by centrifugation in a Centricon 10 Microconcentrator (Amicon, Danvers MA) to 1.5 mg/ml.

Sucrose Gradient Analysis of the AR

Dunning tumor (R3327G) was used as a source of rat AR protein since its receptor undergoes little proteolysis during extraction (7). Tumor was obtained from Copenhagen rats that had received sc tumor transplantations and were castrated 24 h before tumor removal. Tumor cytosol was prepared in buffer containing 10% glycerol, 1 mM diisopropyl fluorophosphate, 10 mM sodium molybdate, and 50 mM Tris, pH 7.2 as previously described (52). A 60% saturated ammonium sulfate fraction was suspended in 10% glycerol, 10 mM mercaptoethanol, and 50 mM Tris, pH 7.2, and incubated with 15 nM [^3H] dihydrotestosterone for 1 h at 4 C. When the antibody titer in crude antiserum was determined, receptor was labeled with 10 nM [^3H]17α-methyltrienolone (New England Nuclear, Boston, MA; 87 Ci/mmol) to avoid interaction with rabbit sex hormone binding globulin. Ammonium sulfate fractions containing labeled receptor were dialyzed for 1 h at 4 C against 10% glycerol, 5 mM mercaptoethanol, 0.15 M KCl, and 50 mM Tris, pH 7.2. Samples were incubated with antibody as described in the legend to Fig. 6 and treated with charcoal to remove unbound steroid before analysis on sucrose gradients. Gradients of 2–20% (wt/vol) sucrose contained 5 mM mercaptoethanol, 10% glycerol, 0.15 M KCl, and 50 mM Tris, pH 7.2. Ovalbumin (3.6S) and γ-globulin (7S) were included as internal molecular weight markers (52) and assayed using the Lowry protein assay (53).

Acknowledgments

Received July 20, 1988. Accepted August 22, 1988.

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Supported by Grants HD-16910, HD-04466, HD-21744, and P30-HD-18968 (recombinant DNA and histochemistry cores) from the National Institute of Child Health and Human Development Center for Population Research, by NIH Grants NS-17479, T32-DK-07129, and by the Andrew W. Mellon Foundation.

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The Endocrine Society, is providing grants for travel and per diem for the 71st Annual Meeting, June 21–24, 1989 in Seattle, Washington. Recipients will be selected on the basis of merit.

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The following restrictions apply.

1) The applicant must be an author on an abstract submitted for the 71st Annual Meeting.
2) Funds are available for travel within the United States or Cananda only.
3) Recipients of 1988 travel grants, scientists holding postdoctoral research positions for more than four years, and undergraduate students are ineligible.